Nanoparticle Delivery of Suicide DNA for Epithelial Ovarian Cancer Therapy

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1 Introduction

The standard treatment for patients with advanced stage epithelial ovarian cancer is optimal surgical debulking followed by chemotherapy with paclitaxel plus a platinum-based therapy (cisplatin or carboplatin). Although ~80% of patients receiving this therapeutic regimen have an initial favorable response, recurrent disease will occur in a majority of cases. Regrettably, there are currently no effective therapies for those patients with advanced-stage ovarian cancer, who either do not respond to initial therapy or for those who develop recurrent disease. There is an immediate need for a more effective treatment for this deadly disease.

Gene therapy holds great promise as an alternative treatment for metastatic ovarian cancer. Metastatic tumors in this disease are nearly always confined to the peritoneal cavity, so intraperitoneal delivery of therapeutic DNA allows for direct treatment of the tumors. In addition, this delivery route protects healthy organs outside the cavity from harmful side effects. In theory, the ability to target the delivery of DNA to tumor cells, as well as the ability to control its expression once inside the cell, provides an added level of therapeutic efficiency and specificity that is difficult to achieve using chemotherapy. In practice, however, the full potential of these advantages of DNA therapies has yet to be achieved and remains a goal of preclinical and clinical studies.

An important consideration in any gene therapy protocol is the choice of vector used to deliver the DNA to cells. With a few exceptions, viral vectors (either adenoviral or retroviral) have been used in clinical trials for the treatment of ovarian cancer (see http://clinicaltrials.gov). Recently, the use of nonviral vectors for the delivery of therapeutic genes is receiving wide attention by the research community, particularly in light of the serious consequences that have occurred in association with the use of viral vectors in patients.

Another consideration in designing a gene therapy protocol is the nature of the DNA that will be delivered. Gene therapy treatment strategies used in ovarian cancer clinical trials include molecular chemotherapy (prodrugs), mutation compensation, immunotherapy, altered drug sensitivity, antiangiogenic therapy, and virotherapy (37, 39).

G. Coukos et al. (eds.), *Ovarian Cancer.*

In this report, we describe suicide gene therapies for epithelial ovarian cancer that we are developing. These therapies are based on the use of a class of cationic polymers called poly(β-amino ester)s to deliver the so-called suicide genes to tumor cells, resulting in their death. Two mouse models for ovarian cancer that are being used in preclinical testing of therapeutic efficacy are also discussed.

2 Suicide Gene Therapy

2.1 Diphtheria Toxin

Targeting the death of ovarian cancer cells, both in the primary tumor and in metastatic lesions, is an attractive therapeutic option. The naturally occurring toxin made by the bacterium *Corynebacterium diphtheriae* is an especially good choice for use as a therapeutic agent because its mechanism of action is known (13), and the gene encoding the toxin has been cloned, sequenced, and adapted for expression in mammalian cells. This toxin is also extremely potent; a single molecule is sufficient to kill a cell (47). Normally, the toxin is secreted as a precursor peptide that is then enzymatically cleaved into two fragments, A and B chains. The B chain binds to the surface of most eukaryotic cells and is required for delivery of the A chain (DT-A), encoding the toxin, into the cytoplasm. Once inside the cell, DT-A inhibits protein synthesis by catalyzing the ADP ribosylation of EF-2 elongation factor. Diphtheria toxin is especially well-suited to treat many kinds of cancer because, unlike virtually all therapeutic agents in use, it kills cells in a cell-cycle independent manner, so that both dividing and nondividing cells are vulnerable to its deadly effects.

A *DT* gene, engineered for use in mammalian cells, *DT-A*, contains the coding sequence for the DT-A subunit, but not for the DT-B subunit (32). DNA constructs can be engineered so that DT-A expression is controlled by cell-specific, *cis*-acting transcriptional regulatory elements (promoters and enhancers). Use of such constructs restricts expression of the DT-A subunit to target cells. In the absence of the B subunit, even DT-A released from dead cells is not able to enter other neighboring cells. This feature thus allows for death of the tumor cells, but not neighboring healthy cells. It also requires efficient uptake of the therapeutic DNA by the tumor cells in order for the therapy to be effective.

A concern in using a toxin as potent as DT-A is the ability to tightly control gene expression. To address this concern, we have developed a novel genetic strategy that makes use of a site-directed recombinase, Flp recombinase (27, 35, 36). Using this strategy, gene expression is regulated both transcriptionally and by DNA recombination mediated by Flp recombinase (Fig. 1). In this system, a cell-specific promoter controls the expression of the recombinase, and so recombinase-mediated recombination event only takes place in selected cells. In those cells where Flp recombinase is expressed, a gene sequence containing the target sequences for the recombinase undergoes excisional recombination, which results

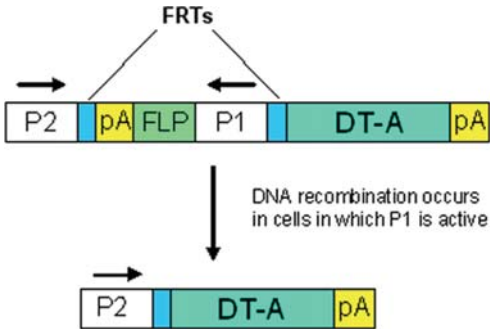

Fig. 1 DNA construct to control DT-A expression. Flp recombinase is produced in tumor cells where P1, a tumor-specific promoter is active. When Flp recombinase is expressed, recombination occurs, allowing a second tumor-specific promoter, P2, to drive expression of DT-A. FRTs are Flp recombinase target sequences. *pA*, polyadenylation sequence. *Horizontal arrows* indicate the direction of transcription

in DT-A expression due to juxtaposition of a second cell-specific promoter to the DT-A coding sequence. Thus, two cell-specific promoters tightly regulate toxin gene expression. By selecting tumor-specific (or tissue-specific) promoters, this system can be applied to target DT-A suicide gene therapy to different kinds of tumors. Delivery of a PSA promoter-regulated DT-A/FLP recombinase DNA construct to human prostate tumor cell xenografts in nude mice resulted in suppression of tumor growth and even tumor regression (6).

In current studies, we are using a promoter sequence of the human mesothelin (*MSLN*) gene to regulate the expression of Flp recombinase. Activity of this promoter is significantly enhanced in ovarian cancer cells relative to normal ovarian cells and cells in other tissues, suggesting its promising use as a cancer-specific promoter in gene therapy strategies (12). The promoter of the gene encoding human epididymis protein 4 (HE4), another promoter having high activity in epithelial ovarian cancer cells, drives expression of DT-A upon DNA recombination (7).

2.2 *CD/5-FC + HSV-TK/GCV Gene-Directed Enzyme Prodrug Therapy*

The efficacy of cytosine deaminase (CD)/5-flurocytosine (5-FC) and herpes simplex virus type 1 thymidine kinase (HSV-TK)/ganciclovir (GCV) suicide gene-prodrug strategies have been studied extensively in animal models and clinical trials (16, 17). In each of these strategies, a normally innocuous prodrug (5-FC or GCV) is converted by a viral or bacterial enzyme to a toxic compound (5-FU or GCVTP), which causes tumor cells to die. In contrast to diphtheria toxin-based therapy, these prodrug therapies are effective primarily against dividing cells. Production of the toxic drug by cells can also cause neighboring cells to die, a so-called “bystander

effect." This may result in the death of more tumor cells, but the trade-off is that it may also result in the death of healthy cells.

Although studies have shown minimal nonspecific toxicity associated with delivery of the genes and administration of the prodrugs, each of these therapies has shown only modest effects on reducing tumor burden and improving clinical outcomes of patients (20). However, when the CD/5-FC and HSV-TK/GCV strategies are administered in combination, therapeutic efficacy is significantly improved when compared with either strategy that is being used alone (1, 8, 19, 40, 46). In a recent report, Boucher et al. demonstrate that sequential administration of the prodrugs (5-FC, then GCV) to cells infected with an adenovirus containing a *CD/HSV-TK* fusion gene in vitro enhanced cytotoxicity above an additive effect by 24- to 35-fold when compared with one to fivefold increase with simultaneous treatment (10). This study suggests that sequential administration of the prodrugs to cancer patients may significantly improve therapeutic outcome.

In preclinical studies in mouse models for ovarian cancer, we are studying the efficacy of sequential prodrug administration following delivery of a *CD/HSV-TK* fusion gene regulated by the MSLN promoter.

3 Poly(β-amino ester)s

The safe and effective delivery of DNA remains a central challenge to the application of gene delivery in the clinic. Currently, the majority of gene therapy protocols employ viral delivery systems, which are associated with serious toxicity and production concerns (43). Nonviral delivery systems offer a number of potential advantages, including ease of production, stability, low immunogenicity and toxicity, and capacity to deliver larger DNA payloads (23). Their use in gene therapy protocols for the treatment of cancer is especially relevant given the low amounts of the requisite receptor for adenoviral infection, CAR, on primary tumors. However, existing nonviral delivery systems are far less efficient than viral vectors (28).

One promising group of nonviral delivery compounds is cationic polymers, which spontaneously bind and condense DNA into nanoparticles (11, 18, 21, 25, 26, 29, 30, 42). A wide variety of cationic polymers that transfect cells in vitro have been characterized; some are natural polymers such as protein (18) and peptide systems (42), while others are synthetic polymers such as poly(ethylene imine) (PEI) (11, 42) and dendrimers (21). Recent advances in polymeric gene delivery have focused in part on the incorporation of biodegradability to decrease toxicity. Typically, these polymers contain both chargeable amino groups, to allow for ionic interaction with the negatively charged DNA phosphate, and a degradable region, such as a hydrolyzable ester linkage. Examples of these include poly[alpha-(4-aminobutyl)-L-glycolic acid] (25), network poly(amino ester) (26), and poly(β-amino ester)s (2, 3, 5, 30). The Langer laboratory has been particularly interested in poly(β-amino ester)s as delivery agents, as they are easily synthesized via the

conjugate addition of a primary amine or bis(secondary amine) to a diacrylate, transfect cells with high efficiency in vitro, and generally possess low toxicity.

The efficacy of various cationic polymers in vivo has been demonstrated both in general and therapeutic models (24). To date, most in vivo work has used PEI. Modification of PEI to include both targeting ligands and serum resistance has been demonstrated and shown to be moderately effective at delivering DNA in a targeted fashion in some systems (9, 22, 44). However, PEI is both nondegradable and relatively toxic, and still not as effective at delivery as viral systems. Recently, attention has turned to the development of cationic polymers that are more compatible with in vivo usage. Using high throughput methods, the synthesis and screening of over 2,350 poly(β-amino ester)s was recently completed (5). This initial screening identified 46 new, biodegradable polymers, which transfect cells as well, and in some cases significantly better than, conventional nonviral delivery system such as PEI in vitro. Subsequent scaled-up resynthesis and analysis of over 500 of these initial polymers has identified the critical importance that polymer molecular weight and end-group termination have on transfection potential (2–4). The polymer that transfects cells most efficiently, C32 (Fig. 2), consistently well-outperforms any commercially available compound tested, and is much less toxic than PEI (45). When C32 complexes with DNA at an optimal polymer/DNA ratio, nanoparticles are formed, which have a molecular weight of 18,100 Da and a diameter of 70 nm (4). Recently, we have shown that intraperitoneal gene delivery using C32 polymers containing end-modified amines results in improved expression levels in several abdominal organs and in ovarian tumors (48). On the basis of these studies, C32 and other polymers similar to it merit further investigation as new vehicles for gene delivery for the treatment of ovarian cancer.

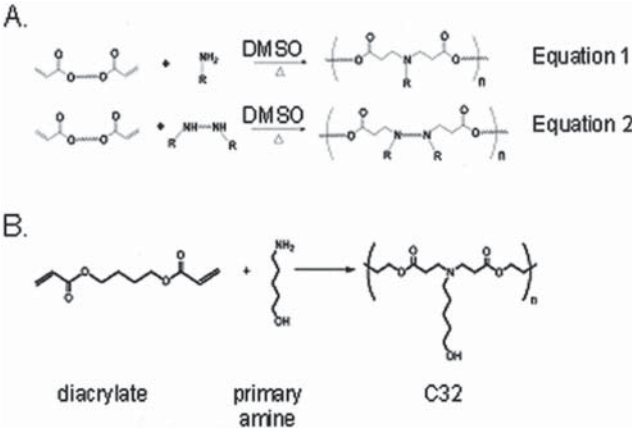

Fig. 2 Synthesis of poly(β-amino ester)s. (**a**) Poly(β-amino ester)s are synthesized by the conjugate addition of primary (*equation 1*) or bis(secondary amines) (*equation 2*) to diacrylates. (**b**) Synthesis of polymer C32

4 The Challenge: Targeted Therapy

Effective treatment of patients with advanced late-stage disease is the major challenge facing clinical oncologists. Although improved detection methods continue to increase the number of patients diagnosed at early stages of disease, the fact remains that, at the time of diagnosis, as many as half of all cancer patients present with metastatic disease. The percentage of ovarian cancer patients presenting with advanced disease is even higher than for other cancers (75%) given the absence of an effective screening for early detection and the relatively asymptomatic nature of the early stages. In addition, many ovarian cancer patients "cured" of their initial malignancy relapse with more aggressive drug-resistant metastatic cancer.

Finding ways to target systemically-delivered therapies to tumor cells and causing minimal toxicity to healthy, nontumorous cells are the key to the development of effective therapies for metastatic disease. Targeting therapy to specific cells can be accomplished by transcriptional targeting, transductional targeting, and ideally, by a combination of both of these approaches.

Transcriptional targeting refers to the use of gene regulatory elements (promoters and enhancers) to restrict gene *expression* to specific cells. The regulatory elements of several ovarian-specific genes have been cloned and characterized. As discussed earlier, we are using two promoter sequences that have enhanced activity in ovarian tumor cells – the promoter of the mesothelin (*MSLN*) gene (12), and the promoter of the gene encoding whey-acidic protein human epididymus protein 4 (*HE4*) (7). Compared with other "ovarian tumor-specific" promoters, these two promoters were recently shown to have the lowest activity in normal tissues (41).

Transductional targeting refers to the *delivery* of DNA to specific cells. Conjugation of vectors (either viral or nonviral) to proteins that have high affinity for specific cells is an effective way to target DNA delivery (15). Proteins that are used for this purpose include ligands, receptors, antibodies, or peptide antagonists. High affinity targeting of the vector to targeted cells will result in reduced sequestration of the particles in nontargeted tissues and more efficient DNA delivery to the targeted population. Successful targeting should reduce the effective dose, thereby reducing any toxicity associated with the therapy.

In our studies, in collaboration with Gregory Adams (Fox Chase Cancer Center), we are conjugating poly(β-amino ester)s with a single chain variable antibody fragment (scFv) of human origin having reactivity to Mullerian Inhibiting Substance II Receptor (MISIIR), a transmembrane serine threonine kinase that is specifically expressed in ovary surface epithelial cells, ovarian tumor cells, in the uterus and Fallopian tubes, and at lower levels in the breast, but not in other tissues in women (31). Its high expression by tumors suggests that it will provide a useful targeting signal for directed therapies.

5 Ovarian Cancer Mouse Models for Preclinical Studies

Mouse models for cancer are very useful for evaluating the efficacy of new gene therapy strategies. Given the importance of identifying possible immunological

responses to treatment, and the importance of the microenvironment on tumor development, transgenic mouse models that have an intact immune system and develop organ-specific cancer are preferred over subcutaneous xenograft models or orthotopic tumors in immunocompromized mice. Importantly, refinement of new imaging modalities for small animals including optical imaging, microCT, PET, and MRI makes it possible to monitor accurately the effect of therapies on tumor development.

Transgenic mouse models for epithelial ovarian cancer that recapitulate human disease have only recently been developed [see review of genetically modified mouse models for ovarian cancer (33)]. We are using the MISIIR/Tag transgenic mouse model, developed by Denise Connolly and Thomas Hamilton, to test the efficacy of nanoparticle-delivered suicide gene therapy (14). As a consequence of expression of the transforming region of SV40 under control of the Mullerian inhibitory substance type II receptor gene promoter, 100% of female MISIIR/Tag mice develop bilateral epithelial ovarian tumors. To evaluate the effect of intraperitoneal administration of nanoparticle-delivered DT-A DNA on tumor growth, mice are CT scanned before treatment and then multiple times after treatment. Amira software is used to generate 3D-reconstructions from tumor images, and tumor volumes are then determined using Image J software (Fig. 3). We are also evaluating the effect of this therapy on the lifespan of MISIIR/Tag mice.

Preliminary studies suggest that the lifespan of MISIIR/Tag mice that receive multiple intraperitoneal injections of poly(β-amino ester) nanoparticles to deliver a MSLN promoter/DT-A DNA is significantly increased when compared with mice treated with control DNA.

A second mouse model we are using employs a cell line, MOSEC, derived from mouse ovarian surface epithelial cells that spontaneously transformed in culture

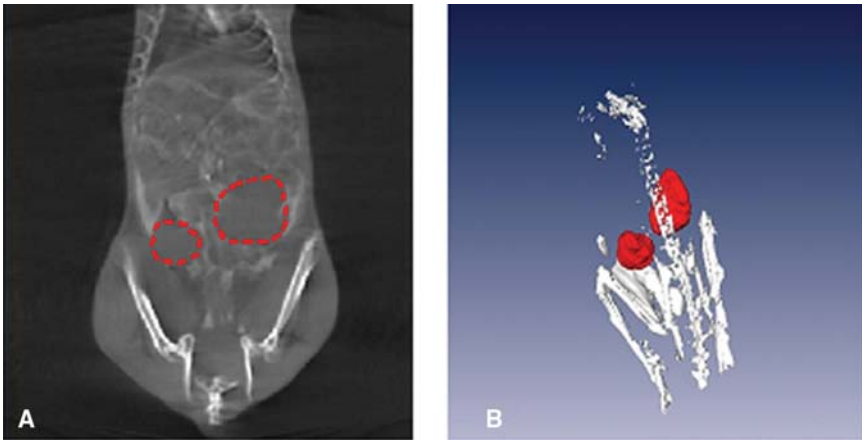

Fig. 3 (**a**) MicroCT scan of a MISIIR/Tag mouse. *Red dashed lines* delineate bilateral ovarian tumors. (**b**) 3-D reconstruction of the tumors in the same mouse

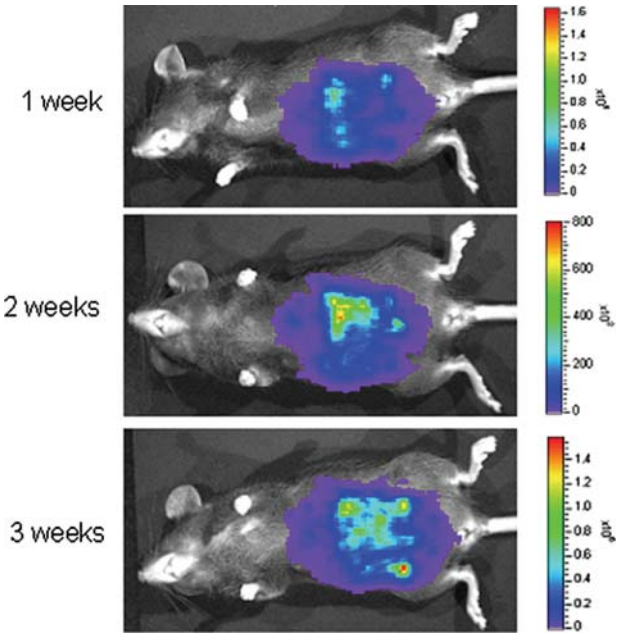

Fig. 4 Optical images of a mouse injected with MOSEC-luc cells taken 1-, 2-, and 3-weeks after injection of the cells into the peritoneum of a C57BL/6 female mouse. Pseudocolor images representing emitted light are superimposed over grayscale reference images of the mouse. RLUs/pixel are indicated in the color scale bar

(38). The properties of this cell line, established by Katherine Roby and Paul Terranova, are described in Chapter 15. When MOSEC cells are injected into the peritoneum of a syngeneic C57BL/6 female mouse, tumors develop throughout the peritoneal cavity. We stably transfected MOSEC cells with DNA encoding firefly luciferase under the control of a strong, ubiquitously expressed promoter/enhancer, CAG (34), and established a clonal cell line, MOSEC-luc. The tumor load of mice injected intraperitoneally with these cells can be quantified by optically imaging these mice to detect bioluminescence following administration of D-luciferin, the substrate of firefly luciferase (Fig. 4). Thus, quantitation of relative light units (RLU) before and after treatment is a convenient and accurate way to assess therapeutic efficacy.

6 Summary

Intraperitoneal administration of polymeric nanoparticles to deliver DNA encoding suicide genes holds much promise as an effective therapy for advanced epithelial ovarian cancer. Poly(β-amino ester)s, a class of cationic, biodegradable polymers

complex to DNA to form nanoparticles that deliver DNA to cells in ovarian tumors. Modifications to poly(β-amino ester)s can improve both the efficiency and specificity with which DNA is delivered to tumor cells. Preclinical studies to test therapeutic efficacy of gene therapy strategies that are under development make use of mouse models for epithelial ovarian cancer and new imaging technologies.

Acknowledgments This work is supported by a grant from the Department of Defense (JAS) and NIH grant EB00244 (DGA and RL).

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